CATHEPSINOGEN D: CHARACTERIZATION AND ACTIVATION TO CATHEPSIN D AND INHIBITORY PEPTIDES

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1. Introduction

It is well known that the extracellular mammalian aspartic proteinases pepsin and chymosin are synthesised in the form of their zymogens which can then be converted by limited proteolysis to the physiologically active forms. In this process amino-terminal activation segment peptides are cleaved off from the zymogen molecule [1–7]. Some of the peptides released during pepsinogen activation can inhibit active enzymes to varying extents [1,5–7]. It has been shown that the conversion of pepsinogen to pepsin at pH < 3 occurs predominantly as a first-order intramolecular activation process [8–10].

In contrast, there are only a few reports concerning the existence of lysosomal enzymes in the form of precursors [11–15]. Among them the main lysosomal aspartic proteinase, cathepsin D, was detected in a higher M_r precursor form during biosynthesis in microsomal vesicles [12,13] and cultured human fibroblasts [14,15]. Some of our studies also indicated the possibility of the existence of cathepsin D zymogen [16,17].

This work reports the isolation of a higher M_r inactive protein which, after brief acid treatment between pH 2.5–3.5, can release cathepsin D and activation peptides. Three of the peptides released show inhibitory activity towards active cathepsin D. We present evidence for the existence of a cathepsin D zymogen, and suggest that it can be activated by a similar mechanism to that involved in the activation of pepsinogen.

Abbreviations: SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass

This paper is dedicated to Helmut Holzer, Professor of Biochemistry, University of Freiburg, FRG, on the occasion of his 60th birthday

2. Materials and methods

Sephadex G-100, Sepharose 4B, AH-Sepharose 4B and protein markers were obtained from Pharmacia Fine Chemicals AB. Pepstatin was purchased from the Peptide Institute, Japan. Cyanogen bromide was from Sigma Chemicals. All other chemicals used were of reagent grade.

2.1. *Isolation of precursor*

Cathepsin D precursor was isolated from bovine spleen by a purification procedure [16,18], with the modification that ammonium sulphate and acetone precipitation steps were omitted. The supernatant (pH 6.3) was directly concentrated by ultrafiltration (Amicon H1P10 Diaflo membranes) to 1/3rd of the initial volume, adjusted to pH 5.0 by the addition of 1/4th the volume of 0.4 M Na-acetate/4 M NaCl (pH 5.0) buffer and 0.1 M acetic acid and then centrifuged at 5000 × *g* for 10 min in a Sorvall refrigerated centrifuge, GSA rotor at 3°C. The clear protein solution containing 30–50 mg protein/ml was immediately applied to pepstatin–Sepharose resin and further chromatographed by gel chromatography on Sephadex G-100 as in [16,18]. Proteolytically active fractions eluted in the first protein peak were pooled, concentrated by ultrafiltration and used as purified cathepsin D precursor.

2.2. *Activation of precursor*

The 5 ml precursor solution (7.2 mg protein) was acidified with 1 M acetic acid to pH 3.5 and immediately applied to the immobilized cathepsin D column (1.3 × 4.0 cm). Cathepsin D was covalently bound to Sepharose 4B by the cyanogen bromide procedure [19]. After loading the sample on to the column, the resin was washed with 0.1 M Na-acetate buffer/0.1 M

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NaCl (pH 3.5) until the released cathepsin D and other non-bound material was eluted completely. Further elution was carried out by the addition of the same buffer at pH 5.0. Later, solutions of 0.1 M NaCl/0.1 M Tris–HCl (pH 6.8) and 0.5 M NaCl/0.1 M Tris–HCl (pH 8.6) were applied to the column. The whole of the activation process was done in the cold room at 3°C.

2.3. *Inhibition studies*

The inhibition of cathepsin D with released inhibitory peptides and pepstatin was determined by cathepsin D assay. A known amount of cathepsin D (0.4×10^{-6} μM) was titrated with increasing amounts of inhibitory peptide and the residual enzyme activity was plotted against mol inhibitor/mol enzyme. For the determination of mol cathepsin D, a specific absorbancy $A_{280}^{1\%}$ of 10.5 was assumed [20].

2.4. *Polyacrylamide gel electrophoresis*

PAGE was done as in [21] using 7.5% acrylamide with 2.5% stacking gels (0.5 × 5.0 cm) in a Tris–glycine buffer (pH 8.9). SDS–PAGE was performed by the method in [22]. Gels (0.5 × 9.5 cm) containing 10% acrylamide were loaded with protein samples dissolved in 1% SDS and 0.1% β-mercaptoethanol. After electrophoresis, gels were stained for protein with Coomassie brilliant blue R-250. The M_r standards were bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400).

2.5. *Other analytical methods*

Cathepsin D activity was determined as in [16]. Protein concentration was determined as in [23]. Peptides were identified by the ninhydrin method [24]. The amino acid analyses were performed with a Beckman model 118 CL amino acid analyzer using 4 N methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce) as in [25]. M_r-Value was determined by gel filtration on Sephadex G-100 by the method in [26] and SDS–PAGE as in [22].

3. Results

Purified cathepsin D precursor was acidified to different pH-values and checked for cathepsin D activity (fig.1). It is evident that the maximal release of proteolytic activity occurred between pH 2.5–3.5.

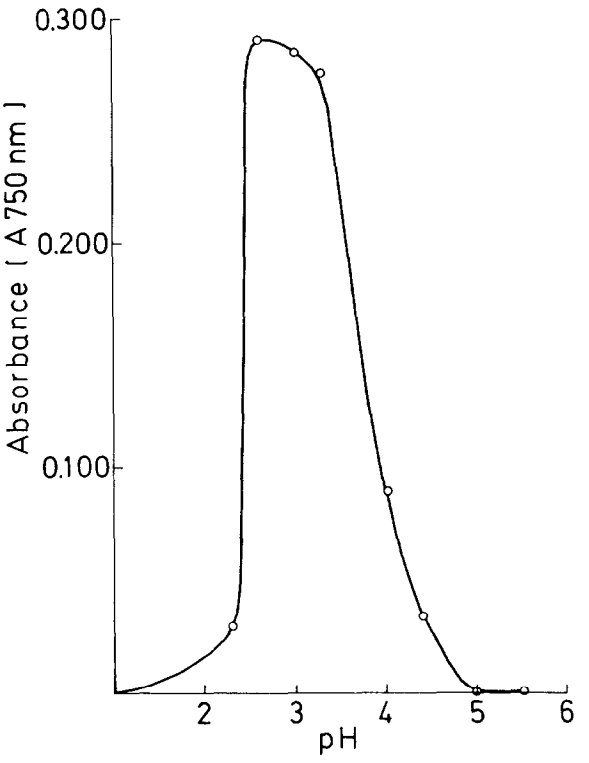

Fig.1. The pH dependence of the release of cathepsin D from its precursor. To 0.05 ml precursor (1.44 mg protein/ml) 0.350 ml buffer was added. From pH 2.3–3.0, 0.1 M citrate buffer and from pH 3.0–5.5, 0.1 M acetate buffer was used. After 2 min preincubation at room temperature the samples were assayed for enzyme activity.

Because of the instability of cathepsin D in this acid region, it is necessary after the activation process to apply the sample to the immobilized cathepsin D column immediately. Immobilized cathepsin D was used for the separation of inhibitory peptides because of its high expected specificity to these released inhibitory peptides. Fig.2 illustrates the elution profile which was obtained. The cathepsin D activity was eluted in the first peak. It should be added that the appearance of the small proteolytically active peak was always detected through several experiments. The inhibitory peptides were bound to the column under these conditions and eluted under changed elution conditions. Three peptides were eluted and fractions were assayed for inhibitory activity. From the elution profile, the successful separation of three inhibitory peptides designated A, B and C, respectively, is evident. Whereas the first two peptides show some

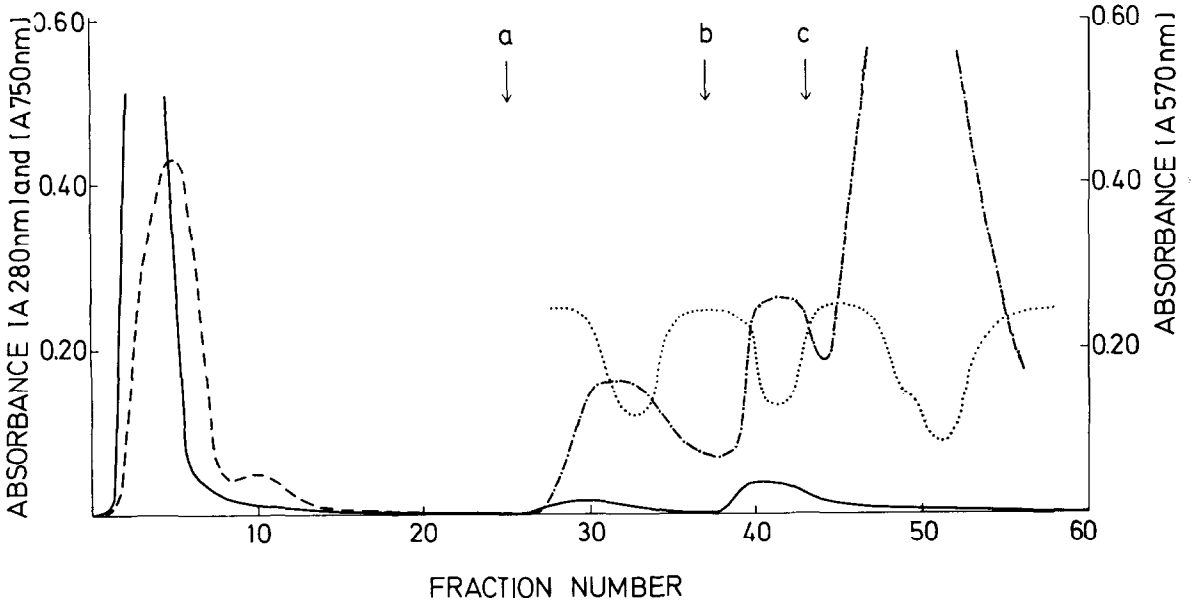

Fig.2. Affinity chromatography of activated cathepsinogen D on immobilized cathepsin D–Sepharose 4B resin. Arrows a, b and c indicate the elution buffers changes to pH 5.0, pH 8.6 in 0.1 M NaCl and pH 8.6 in 0.5 M NaCl, respectively. Fractions of 2.5 ml were collected and 0.2 ml was assayed for enzyme activity, ninhydrin reaction. Peptide inhibition: 0.2 ml fraction was added to 0.2 ml cathepsin D sample, preincubated at room temperature for 10 min and assayed for the remaining enzyme activity. Spectrum of: (——) protein A_{280}; (– – –) cathepsin D activity, A_{750}; (—·—·) peptide (ninhydrin reaction A_{570}); (. . .) inhibitory activity, A_{750}.

absorbance at 280 nm, peptide C shows practically no absorbance in this region, indicating a very low content of aromatic acid residues or their absence. In fig.3 the polypeptide composition of purified cathepsin D precursor on SDS–PAGE, besides a protein band in the upper part of the gel (under investigation) shows the presence of a polypeptide chain of $M_r \sim 30\,000$ and a faint band $M_r \sim 16\,000$ (gel C), indicating the presence of cathepsin D polypeptide chains (gel B). PAGE of activated cathepsin D gives practically a single band which corresponds to one of the multiple forms of cathepsin D (gel D). Additionally, M_r of ~ 45 000 for activated cathepsin D was

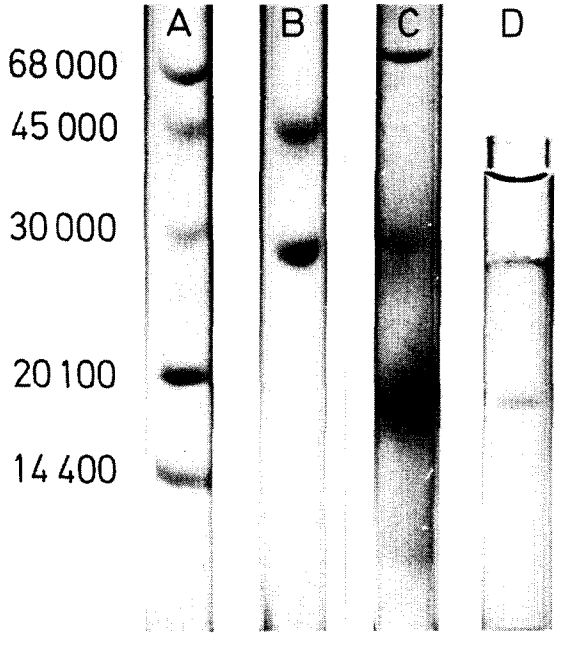

Fig.3. Polyacrylamide gel electrophoresis. In the presence of SDS and β-mercaptoethanol: (A) marker proteins; (B) two polypeptide chains of cathepsin D (M_r 45 000 and ~29 000–30 000); (C) polypeptide composition of cathepsinogen D before activation; (D) at pH 8.9, the released cathepsin D.

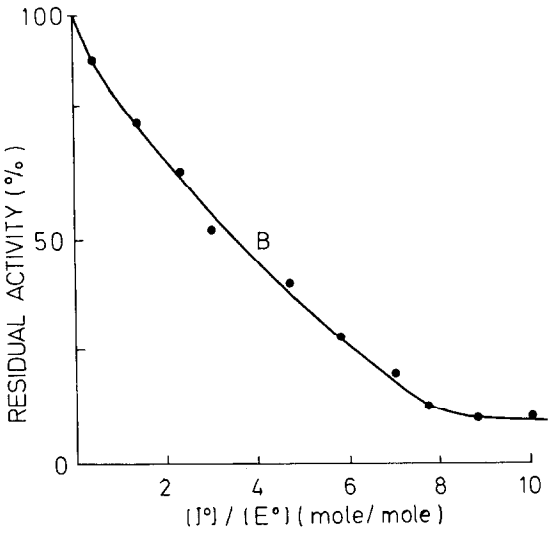

Fig.4. Interaction of cathepsin D with the released inhibitory peptide from cathepsinogen D. Increasing amounts of a solution, containing inhibitory peptide B (starting from 0.025 ml) were added to known amount of cathepsin D (0.4×10^{-6} μM) and the remaining enzyme activity was measured.

obtained by gel chromatography on Sephadex G-100 (not shown). The pentapeptide pepstatin, the most powerful inhibitor of aspartic proteinases, completely inhibits cathepsin D activity at 10^{-9} M (mol/mol).

Increasing amounts of peptide B were added to the known amount of cathepsin D in the assay and the percentage inhibition was calculated at each concentration of inhibitor. The result presented in fig.4 shows that ~7 mol inhibitory peptide B is needed for complete inhibition of 1 mol cathepsin D. The concentration of the inhibitory peptide was calculated from the amount of the released cathepsin D from its zymogen, assuming that 1 mol peptide is liberated/1 molecule cathepsin D.

The amino acid composition of the activation peptide B is presented and compared with that found in pig and bovine pepsinogen (table 1). There is evidently a similarity between cathepsinogen D activation peptide B and the activation segment obtained from pig pepsinogen and bovine pepsinogen residues 18–45.

Table 1
Amino acid composition (mol residue/mol) of bovine cathepsinogen D inhibitory peptide B, pig and bovine pepsinogen activation peptides

Amino acid	Bovine cathepsinogen D[a] found	Pig pepsinogen[b]		Bovine pepsinogen[c]	
		Activation peptide, found	Residues 1–44	Residues 1–45	Residues 18–45
Lys	2.31 (2)	4.60	9	8	4
His	1.00 (1)	1.34	2	1	1
Arg	1.37 (1–2)	1.14	2	3	2
Asp	2.98 (3)	4.00	4	3	2
Thr	1.82 (2)	1.28	1	2	2
Ser	1.31 (1–2)	2.16	2	3	1
Glu	2.52 (3)	2.52	2	4	3
Pro	1.38 (1)	2.26	3	1	0
Gly	4.14 (4)	2.20	1	2	2
Ala	2.23 (2)	2.88	4	2	2
Val	1.29 (1)	2.20	3	3	0
Met	0.68 (1)	0.00	0	1	1
Ile	1.46 (1–2)	1.32	1	3	2
Leu	2.13 (2)	4.85	7	6	3
Tyr	1.17 (1)	0.95	1	2	2
Phe	0.86 (1)	1.36	2	1	1
Total	27–30		44	45	28

[a] Time-course hydrolysis: 24 h and 48 h; [b] See [3,5]; [c] See [4]

4. Discussion

The rapid purification procedure which we have developed for cathepsin D and which includes affinity chromatography on pepstatin–agarose [18] enabled us to isolate both cathepsin D and an inactive protein which represents a cathepsin D zymogen, cathepsinogen D. This zymogen binds to immobilized pepstatin at pH 5.0 and is recovered by elution at higher pH in a similar fashion to that reported for porcine pepsinogen [10] and cathepsin D isozymes from porcine spleen [27]. While activation of the zymogen has been shown to take place rapidly at pH 3.5, this process is minimized by exposing it to these conditions for a brief time only and at 3°C. It is still possible, however, that a large proportion of the precursor is converted to active enzyme at this point in the isolation procedure and that the yield of the precursor obtained here does not represent the total amount in the tissue. Many other purification procedures for cathepsin D include an autolysis step (review [28]) and this may be one of the reasons why the zymogen had not been isolated. The activation of cathepsinogen D is pH-dependent, like that for pepsinogen [4,5]. However, unlike pepsin, cathepsin D is unstable at pH < 4.0 [28] so that the enzyme must be exposed to low pH for as short a time as possible. After activation, three peptides were bound to immobilized cathepsin D while active enzyme and other inactive material including possible non-inhibiting peptides released during the activation process, passed through the column. The bound peptides were eluted separately under different elution conditions indicating differences in their ability to bind the enzyme. These peptides inhibit cathepsin D activity and this phenomenon may account for the earlier report that cathepsin D activity was increased after gel filtration [16]. While electrophoresis indicates the presence of cathepsin D in the inactive protein, the M_r of the activated enzyme and its inhibition by pepstatin proved the release of cathepsin D from the inactive form. It was recently reported that a cathepsin D precursor of M_r 53 000 synthesized in human fibroblasts is inactive and can be processed through intermediate steps to M_r 31 000 and 14 000 polypeptide chains [14].

The amino acid composition of the released peptide B and its N-terminal amino acid, Ile (preliminary result), suggests a close similarity with the pepsinogen activation process. There is a high probability that this peptide of ~27 amino acid residues, with $M_r \sim 3000$, corresponds to the activation peptide which is released during cleavage of the Leu 16–Ile 17 bond in pig and Leu 17–Ile 18 in cow pepsinogen, as summarized in [4,5]. The lower yields of some amino acids found in the pig pepsinogen activation segment, compared to intact residues of 1–44 of pig pepsinogen (table 1) may be due to their having been released as small peptides [3]. It should be added at this point that it has been impossible to isolate the intact activation segment of ~45 amino acid residues from pepsinogen molecule during the activation process. Therefore, we can assume that also in our experiments we were not able to isolate the intact cathepsinogen D activation peptide of ~45 residues with $M_r \sim 5000$. In [13], an activation peptide of 44 amino acids was removed from procathepsin D soon after synthesis in vivo, yielding enzymatically active cathepsin D in a single chain of M_r 44 000 which undergoes further cleavage into an enzymatically active two-chain form.

From the measurements of the inhibition of cathepsin D with the released peptide we can assume that the dissociation constant of the complex is moderate. Several inhibitory peptides were released during porcine pepsinogen activation with different apparent dissociation constants for the peptide–pepsin interaction [6]. These results suggest therefore, that the activation of cathepsinogen D is similar to pepsinogen activation, which occurs predominantly as an intramolecular process at acid pH releasing activation peptides.

Ten years ago the existence of a precursor of cathepsin D from chicken liver was suggested [29]. Later the isolation of a high M_r cathepsin D isozyme of $M_r \sim 100\,000$ with low specific activity was reported [27] and it was suggested that this is the precursor form which can be converted to a two-chain enzyme. Our results show that the activation of cathepsinogen D occurs through a different process. From the M_r of cathepsin D and of the activation peptide we can conclude that cathepsin D precursor has an M_r of ~50 000 and can be converted by intramolecular activation to the active enzyme and activation segment-peptide(s). Further studies are in progress.

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